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Retinoic Acid Receptors exhibit Cell-autonomous Functions in Cranial Neural Crest Cells

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Abbreviations: BA, branchial arch; E, embryonic day; NCC, neural crest cells; RA, retinoic acid; ALDH1A, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RXR, rexinoid receptor, VAD, vitamin A-deficient.

Key words: retinoic acid; branchial arch; craniofacial development; apoptosis; conditional mutagenesis; mouse.

Running title: Retinoic acid receptors in cranial and branchial arch neural crest cells

ABSTRACT

Previous work has emphasized the crucial role of retinoic acid (RA) in the ontogenesis of the vast majority of mesenchymal structures derived from the neural crest cells (NCC), which migrate through, or populate, the frontonasal process and branchial arches. Using somatic mutagenesis in the mouse, we have selectively ablated two or three retinoic acid receptors (i.e., $RAR\alpha/RAR\beta$, $RAR\alpha/RAR\gamma$ and $RAR\alpha/RAR\beta/RAR\gamma$) in NCC. By rigorously analysing these mutant mice, we found that survival and migration of NCC is normal until gestational day 10.5, suggesting that RAR-dependent signalling is not intrinsically required for the early steps of NCC development. However, ablation of *Rara* and *Rarg* genes in NCC yields an agenesis of the median portion of the face, demonstrating that $RAR\alpha$ and $RAR\gamma$ act cell-autonomously in post-migratory NCC to control the development of structures derived from the frontonasal process. In contrast, ablation of the three *Rar* genes in NCC leads to less severe defects of the branchial arches derived structures compared to *Rar* compound null mutants. Therefore, RARs exert a function in the NCC as well as in a separated cell population. This work demonstrates that RARs use distinct mechanisms to pattern cranial NCC.

INTRODUCTION

The morphogenesis of the craniofacial region results from complex developmental processes involving the migration of neural crest cells (NCC) and their subsequent differentiation through interactions with endoderm and ectoderm (Veitch et al., 1999; Trainor and Krumlauf, 2001). Cranial NCC arise from fore-, mid- and hindbrain in distinct migratory streams. Fore- and midbrain NCC give rise to the frontonasal skeleton, whereas pre-otic hindbrain populations colonize the first two branchial arches (BA), where they differentiate to form skeletal elements of the jaw, middle ear and tongue. Cardiac NCC participate in the development of aortic arch arteries, as well as thymus, thyroid and parathyroid glands (Le Douarin et al., 1993; Santagati and Rijli, 2003). Since patterning and morphogenesis of these cranio-facial structures require a finely orchestrated series of tissue interactions (Graham and Smith, 2001; Trainor and Krumlauf, 2001), perturbation of different processes may yield similar developmental abnormalities. For instance, inactivation of genes expressed in either NCC, endoderm or ectoderm, results in similar cardiovascular defects, as remodelling of BA arteries into the definitive arterial vessels and heart outflow tract requires coordinated communication between NCC, endoderm and ectoderm (Depew et al., 1999; Lindsay et al., 2001; Abu-Issa et al., 2002; Stalmans et al., 2003).

Malformations of cranial NCC-derived structures are generated upon impairment of retinoic acid (RA) signalling in various vertebrate species (Lohnes et al., 1994; Mark et al., 1998; Schneider et al., 2001; Jiang et al., 2002a; Jiang et al., 2002b; Kopinke et al., 2006; Halilagic et al., 2007; Vieux-Rochas et al., 2007). The effects of RA on embryonic development are mediated by heterodimers between RA receptor (RAR) isotypes ($RAR\alpha$, β and γ) that are widely expressed in the embryo (Mollard et al., 2000; Dollé, 2009) and retinoid receptors (RXRs) (Chambon, 1996; Mark et al., 2009). The requirement of RARs for pattern formation, differentiation, proliferation and apoptosis during organogenesis has been investigated through generation and analysis of *Rar*-null mouse models (Mark et al., 2009). These studies have shown that *Rara/g*-null mutants display malformations in a large variety of structures derived from cranial and cardiac NCC (Lohnes et al., 1994; Mendelsohn et al., 1994; Ghyselinck et al., 1997). In particular, structures derived from the frontonasal process are agenetic in these mutants, resulting in a median facial cleft. In *Rara/g*-null and *Rara/b*-null mutants, BA2 or BA3 to BA6 are hypoplastic and the laryngeal cartilages are malformed (Mendelsohn et al., 1994; Dupé et al., 1999b; Wendling et al., 2001). The aortico-pulmonary

septum and derivatives of the 3rd, 4th, and 6th aortic arches are also consistently altered in these mutants, leading to persistent truncus arteriosus and to a variety of abnormal arterial patterns (Mendelsohn et al., 1994; Jiang et al., 2002a). In addition, various forms of parathyroid or thymic ectopia or agenesis are observed (Mendelsohn et al., 1994; Ghyselinck et al., 1997). Altogether, these observations led to the proposal that cranial NCC giving rise to mesenchymal derivatives are major targets of RA action (Mark et al., 1998).

While previous work has demonstrated that RA is produced by the ventral epithelium of the frontonasal process and then targets the cranial NCC (Schneider et al., 2001), the question whether RARs have their functions in both cranial NCC and craniofacial ectoderm is still unclear. In addition, various data favoured the involvement of interactions between the arch endoderm and the NCC in the patterning of BAs (Graham et al., 2005). In experimental approaches where RA signalling is impaired, hypoplasia of BA3-4 is always correlated with changes in expression of genes that are crucial for the normal patterning of BAs, notably *Hoxa1* and *Hoxb1* in NCC and endoderm, as well as *Pax1* and *Pax9* in endoderm (Peters et al., 1998; Dupé et al., 1999b; Wendling et al., 2000; Vermot et al., 2003; Mark et al., 2004). These data strongly suggest that RA exerts at least part of its action in the endoderm. Furthermore, a previous study using specific inactivation of RXR α in the NCC suggests that the persistent truncus arteriosus phenotype would be non cell-autonomous for the NCC (Jiang et al., 2002a). However, the actual involvement of each of the RARs in NCCs versus other craniofacial tissues has not been deciphered. The mouse knockout models that have been analyzed so far did not discriminate between direct effects of RARs in a given NCC population and indirect effects mediated by RARs in neighbouring cell populations. In the present work, we have investigated the phenotypes of somatic mutants lacking RAR α , RAR β and/or RAR γ , selectively in NCC and demonstrated that RARs exerts a dual function during craniofacial development. We show that within the frontonasal mass, RAR α and RAR γ act cell-autonomously in NCC to determine the morphogenesis of NCC-derived elements. In contrast, RARs appear mostly dispensable in cranial NCC giving rise to BA2-6 derived tissues, suggesting that they predominantly exert their role in another embryonic cell population such as the BA endoderm.

METHODS

Mice. All mice, with a mixed C57BL/6–129/Sv (50%:50%) genetic background, were housed in an animal facility licensed by the French Ministry of Agriculture (agreement N°B67–218–5), in compliance with the European legislation on care and use of laboratory animals. Heterozygous mice were mated overnight, and animals with a vaginal plug at noon of the next day were considered as embryonic day (E) 0.5. The generation and genotyping of *loxP*-flanked *Rara* (*Rara*^{+/*L2*}), *Rarb* (*Rarb*^{+/*L2*}) and *Rarg* (*Rarg*^{+/*L2*}) have been described (Chapellier et al., 2002a; Chapellier et al., 2002b; Chapellier et al., 2002c). The *Wnt1-Cre* mouse, which carries a transgene containing a *Cre* cassette under the control of the *Wnt1* promoter, and the *R26R* reporter mouse, which expresses *E. coli* β -galactosidase upon *Cre*-mediated excision of a *loxP*-flanked intervening DNA sequence, were obtained from A. McMahon and P. Soriano, respectively (Danielian et al., 1998; Soriano, 1999). The *Wnt1-Cre*, *Rara*^{+/*L2*}, *Rarb*^{+/*L2*} and/or *Rarg*^{+/*L2*} parental lines were intercrossed to generate mutant embryos and fetuses lacking RAR α and RAR β or RAR α and RAR γ in NCC (hereafter designated as *Rara/b*^{NCC-/-} and *Rara/g*^{NCC-/-}, respectively) and lacking all three RARs in NCC (hereafter designated as *Rara/b/g*^{NCC-/-} mice). Littermate embryos without the *Wnt1-Cre* transgene were used as controls (hereafter designated as WT). All mutants were obtained at a Mendelian ratio at birth.

Phenotyping of embryos and fetuses. For histology, samples were fixed in Bouin's fluid for 5 days, embedded in paraffin, serially sectioned and stained with Mallory's trichrome (Mark et al., 1993). Skeletons were stained with alcian blue and alizarin red. For β -galactosidase activity, detection was performed as described (Rossant et al., 1991). In situ RNA hybridization was carried out as described (Matt et al., 2003). The digoxigenin-labeled antisense riboprobes were synthesized using cDNA templates (references upon request).

Terminal transferase-mediated dUTP-Nick-End-Labeling (TUNEL) was performed using the Apoptag® kit (Chemicon International) on 7 μ m-thick serial frontal sections from paraffin-embedded embryos.

Cell proliferation was assessed on 7 μ m-thick serial histological sections using the anti-phosphohistone H3 antibody (Sigma). The number of positive cell nuclei was determined by counting more than 2000 frontonasal cells on each section at the rate of 20 sections by embryo.

RESULTS

Ablation of the three RARs in NCC does not alter their migration

We first tested whether inactivation of all three RARs in the neural crest lineage (i.e., in *Rara/b/g*^{NCC-/-} mutants) altered migration of NCC from the neurectoderm into the frontonasal process and BAs. For this purpose, we used the reporter line (*R26R-LacZ*), which is expressed in the progeny of the NCC that have undergone Cre-mediated recombination (Jiang et al., 2000). At E9.5, both mutant and WT embryos possessed 4 BAs, three of which (BA1 to BA3) have externally visible bulges (Fig. 1a,b). Importantly, the pattern of β -galactosidase activity, identifying NCC in the frontonasal process and BAs, was identical in E9.5 *Rara/b/g*^{NCC-/-} mutants and in control *Wnt1-cre/R26R* embryos (Fig. 1a-f). At E9.5, normal BAs consisted of a core of paraxial mesoderm centred by an aortic arch artery, surrounded by NCC and covered externally with ectoderm and internally with endoderm (Fig. 1e). This BA organization was present in *Rara/b/g*^{NCC-/-} embryos, with histologically normal endoderm and ectoderm (n=2; Fig. 1e-f). To further analyse the migration and patterning of NCC in mutant embryos, we performed whole mount *in situ* hybridizations using two markers of migrating NCC, *Crabp1* and *Dlx5* (Dencker et al., 1990; Depew et al., 1999). We found a normal distribution of *Crabp1* and *Dlx5* in E9.5 *Rara/b/g*^{NCC-/-} mutants (Fig. 1g-j). Lastly, to confirm that segmentation of the BAs occurred correctly in *Rara/b/g*^{NCC-/-} mutants, we analysed the distribution of *Pax1* transcript, which is expressed in the pharyngeal pouch endoderm (Deutsch et al., 1988). As shown in figure 1, *Pax1* was normally expressed in E9.5 *Rara/b/g*^{NCC-/-} mutants (n=2; Fig. 1k-l). Altogether, these data clearly indicate that ablation of all RARs in NCC affect neither their specification and migration, nor the formation of the BAs.

Ablation of the three RARs in NCC does not alter the development of cranial nerve ganglia

To assess whether removing all three RARs in NCC affected the development of cranial nerves, we compared the axonal paths using antineurofilament immunostaining in E10.5 WT and *Rara/b/g*^{NCC-/-} mutants. Additionally, we analysed the expression of *Sox10*, a marker of the neurogenic NCC (Kuhlbrodt et al., 1998). We detected identical patterns of neurofilament distribution and of *Sox10* expression in WT and *Rara/b/g*^{NCC-/-} embryos at E10.5 (n=2; Fig.

1m-p). These data indicate that ablation of RARs in NCC does not alter the fate of their neural derivatives.

RAR α and RAR γ function in cranial NCC is necessary for the formation of most of the frontonasal skeletal elements

At E9.5, *Rara/b/g*^{NCC-/-} mutants were morphologically normal (Fig. 1a-b). However, at birth, *Rara/b/g*^{NCC-/-} mutant mice displayed a truncated face and a mid-facial cleft (n=6; Fig. 2c,d), similar to that described in *Rara/g*-null mutants although exencephaly was not observed (Lohnes et al., 1994). Skeletal analysis of E18.5 *Rara/b/g*^{NCC-/-} fetuses revealed agenesis or malformations of NCC-derived craniofacial bones and cartilages (Noden, 1988; Le Douarin et al., 1993; Chai and Maxson, 2006; Gross and Hanken, 2008; McBratney-Owen et al., 2008; Yoshida et al., 2008). Large portions of frontal bones were missing; the premaxilla, palatal process of premaxilla and nasal cartilage were rudimentary; the upper incisors were absent, the presphenoid bone hardly distinguishable, and the basisphenoid bone was deformed in its anterior part. In contrast, the more posterior structures derived from paraxial mesoderm, i.e. the different parts of the occipital (basi-, exo-, supraoccipital) bone were normal (Table 1; Fig. 2e-i). However, the parietal bone also derived from the mesoderm was reduced (Fig. 2i). The interparietal bone that has a dual origin: its medial portion being crest-derived, and its lateral portion mesoderm-derived (Jiang et al., 2002b), was normal in *Rara/b/g*^{NCC-/-} fetuses (Fig. 2i). *Rara/b/g*^{NCC-/-} mutants also exhibited a supernumerary skeletal element previously detected in *Rara/g*-null mutants, separating the trigeminal ganglion from the brain. This element corresponds to a remnant of the pila antotica, a structure that was lost during evolution from reptiles to mammals (Table 1, data not shown) (Lohnes et al., 1994; Ghyselinck et al., 1997; Mark et al., 1998). Analysis of *Rara/g*^{NCC-/-} fetuses revealed an extensive loss of NCC-derived craniofacial skeletal elements undistinguishable from that seen in *Rara/b/g*^{NCC-/-} mutants. Contrasting with the multiple frontonasal skeletal deficiencies seen in *Rara/g*^{NCC-/-} and *Rara/b/g*^{NCC-/-} mutants, alteration of a single skeletal element was observed in *Rarg*^{NCC-/-} mutants, corresponding to a severe hypoplasia of the presphenoid bone (fig2j-k). *Rara/b*^{NCC-/-} mutants did not show abnormalities in the frontonasal region (Table 1). Altogether, these observations indicate that RAR α and RAR γ act cell-autonomously within the frontonasal mass NCC-derived mesenchyme to direct the morphogenesis of skeletal elements, whereas RAR β is not involved in this process.

All derivatives of the first and second BA are present in *Rara/b/g*^{NCC-/-} mutants

In *Rara/b/g*^{NCC-/-} mutants, the dentary (mandibular) bone, Meckel's cartilage and malleus, all deriving from the mandibular portion of the BA1, were normal (Table 1 and Fig. 2e-i). Conversely, the maxillary bones, as well as the alisphenoid, squamosal, incus, tympanic bone and gonial bone, were present although misshapen. The maxillary, the alisphenoid and the squamosal bone were reduced (Table 1 and Fig. 2e-i). The short process of the incus (SI) was conspicuously larger than in WT, a defect also observed in all *Rara*^{NCC-/-} mutants (Fig. 2l). The tympanic ring was shortened and the gonial bone distorted. This atypical gonial bone was also observed in *Rara/b*^{NCC-/-} mutants; it was the only skeletal malformation associated to the inactivation of both *Rara* and *Rarb* in NCCs.

A pterygoquadrate element (i.e., a skeletal element connecting the alisphenoid bone and the incus) was never observed in *Rara/b/g*^{NCC-/-} mutants (Table 1), whereas it was found in *Rara/g*-null and *Rara/b*-null mutants (Lohnes et al., 1994; Ghyselinck et al., 1997). The cartilaginous derivatives of the 2nd branchial arch, i.e. the stapes, the styloid and the lesser horn of the hyoid bone (Noden, 1988; Le Douarin et al., 1993; Santagati and Rijli, 2003) were normal in *Rara/b/g*^{NCC-/-} mutants (Table 1). The otic capsule, which has a dual embryological origin from ectoderm and NCC (Torres and Giraldez, 1998; O'Gorman, 2005) was also normal. Agenesis of the pinna (the externally visible portion of the outer ear) was never observed in *Rara/b/g*^{NCC-/-} mutants (Fig. 2a-c) although it was found in *Rara/g*-null mutants (Lohnes et al., 1994). Thus, the mild craniofacial skeletal defects of the 1st BA maxillary derivatives previously seen in *Rara/g*-null mutants were also observed in *Rara/b/g*^{NCC-/-} mutants. However, the severe craniofacial defects of the 1st BA mandibular derivatives and of the 2nd BA derivatives observed in *Rara/g*-null mutants were not recapitulated in *Rara/b/g*^{NCC-/-} mutants (Table 1). Altogether, these data indicate that RARs are not involved in NCC for the initial specification of the 1st and 2nd BA derivatives, but rather play a role later in the morphogenesis of some skeletal elements of the 1st and 2nd BA derivatives.

Ablation of RARs in NCC yields relatively few defects in tissues derived from the post-otic BA

We next investigated the status of the great cephalic arteries (i.e., arch of the aorta, subclavian and common carotid arteries), the aorticopulmonary septum, the thymus and the parathyroid glands, as their normal development is supported by the cardiac NCC and they were found to

be severely altered and/or deficient in *Rara/b*- and *Rara/g*-null mutant mice (Lohnes et al., 1994; Mendelsohn et al., 1994; Ghyselinck et al., 1997). About half of the *Rara/b/g*^{NCC-/-} mutants (3 out of 7) displayed a patterning defect of the great arteries consisting of an abnormally positioned aortic arch on the right side of the midline and associated with a persistent truncus arteriosus (PTA) and an interventricular septal defect (Fig. 3a, b and Table 2). Along the same lines, one third of the *Rara/g*^{NCC-/-} mutants, exhibited a retroesophageal subclavian artery (2 out of 6; Table 2). In contrast, *Rara/b*^{NCC-/-} mutants never exhibited defects of the great arteries (n=6). Notably, *Rara/b/g*^{NCC-/-}, *Rara/g*^{NCC-/-} and *Rara/b*^{NCC-/-} mutants never displayed developmental defects of the thymus and thyroid derived from the posterior branchial arch arteries (Table 2). However, the parathyroid glands were always found more rostrally than expected, i.e. never associated with thyroid gland, in *Rara/b/g*^{NCC-/-} mutants at E18.5 (n=3; Fig. 2j-l). One can also note that, despite a normal shape of the hyoid bone, a fusion between the hyoid greater horn and the rostral horn of the thyroid cartilage was consistently observed in *Rara/g*^{NCC-/-} and *Rara/b/g*^{NCC-/-} mutants (Fig. 3c-d). Altogether, these data indicate that RAR α and RAR γ , but not RAR β , have a function in NCC for the proper formation of the post-otic BA derivatives.

Ablation of RAR α and RAR γ alters the apoptotic pattern in the frontonasal area

In E10.5 *Rara/b/g*^{NCC-/-} mutants, the nasolateral and nasomedial processes were present on either side of the olfactory pit. However, at E11.5, these processes are spaced more laterally than in the WT situation (compare double-arrows in Fig. 4). The abnormally wide spacing became more pronounced later during development and result in defective merging of the nasomedial processes in the midline. These observations suggest that ablation of RARs in NCC impacted the medial expansion of the nasal processes, possibly through regulation of cell proliferation or apoptosis. Cell proliferation was assessed at E10.5 and E11.5 using an anti-phosphohistone H3 (PHH3) antibody (a marker of the M-phase of the cell cycle). In the frontonasal mesenchyme of E10.5 WT, the number of PPH3-positive cells was 132 \pm 15 for 2000 counting cells (n=4) versus 144 \pm 17 (n=2) in *Rara/b/g*^{NCC-/-} mutants. At E11.5 (Fig. 5a,e), the number of PPH3-positive cells in the frontonasal region of the WT (n= 4) was 160 \pm 22 versus 154 \pm 25 in *Rara/b/g*^{NCC-/-} mutants (n=3). Therefore cell proliferation in the frontonasal region was not significantly affected by ablation of RARs in the NCC.

The frontonasal region of E10.5 WT embryos displayed, similarly to E11.5 embryos, a cluster of TUNEL-positive cells in the dorsal region of the nasal process (arrowheads in Fig. 5b,d).

Apoptosis was assessed at E10.5 and E11.5 on serial histological sections. At E10.5, out of 30 sections through the entire frontonasal region the apoptotic cluster was consistently observed in WT and *Rara/b/g*^{NCC-/-} mutants (n=3; Fig. 5b,f). Therefore, at E10.5, no significant change was observed for the distribution of apoptotic cells in the frontonasal region of *Rara/b/g*^{NCC-/-} mutants. In E11.5 WT embryos, the large cluster of TUNEL-positive cells located dorsally to the nasal pits was detected on at least 35 consecutive serial sections (n=7; arrowheads in Fig. 5d). In *Rara/b/g*^{NCC-/-} mutants (n=2; Fig. 5h) and *Rara/g*^{NCC-/-} mutants (n=2), this cluster was not detected. Only a few scattered apoptotic cells were observed (arrows in Fig. 5h). Altogether, these data indicate that apoptosis in the frontonasal mesenchyme is controlled by RAR α and RAR γ . At E11.5, we also identified on WT embryos a single cluster of apoptotic cells located on the midline (n=7; brackets in Fig. 5c), which was replaced by two smaller apoptotic clusters on each side of the midline in *Rara/b/g*^{NCC-/-} (n=2) and *Rara/g*^{NCC-/-} mutants (n=2; Fig. 5g). This difference can be accounted for by the abnormal wide space between the 2 nasomedial processes in *Rara/b/g*^{NCC-/-} mutants.

DISCUSSION

Although, the crucial role of RARs in NCC-derived craniofacial structures has been established for years (Lohnes et al., 1994; Ghyselinck et al., 1997; Mark et al., 2009), evidence that this role could be cell autonomous was still lacking. The difficulty of understanding the functions of RARs in NCC originated from several problems. Firstly, conventional germline RAR compound null mutants display a broad spectrum of abnormalities affecting a variety of organs constituted by cells of multiple origins. Secondly, RARs exhibit widespread and rapidly changing expression patterns in the developing embryo (Dollé, 2009), and RA is a secreted molecule which can act in a paracrine manner (Matt et al., 2005; Duester, 2008; Matt et al., 2008; Niederreither and Dollé, 2008). To overcome these difficulties and to gain insights into the functions of RA, it was necessary to produce and analyse mice in which RARs were specifically ablated in NCC.

RAR α and RAR γ exert cell-autonomous function in frontonasal NCC

The present work shows that somatic inactivation of *Rara* and *Rarg* in NCC (i.e., *Rara/g*^{NCC-/-} mutants) phenocopies almost all the abnormalities displayed by the corresponding germline null mutants (i.e., *Rara/g*-null mutants) in the frontonasal skeletal elements, namely the

frontal, nasal, premaxillary, presphenoid, and basisphenoid bones, as well as in the upper incisors (Lohnes et al., 1994). This demonstrates that RAR α and RAR γ are cell-autonomously implicated in the development of the NCC-derived frontonasal skeletal elements. Besides, as *Rarg*^{NCC-/-} mutants display severely reduced presphenoid bones, RAR γ is predominantly involved in the formation of this bone.

Additionally, the supraoccipital and interparietal bone appear normal in *Rara/g*^{NCC-/-} mutant mice, while they are severely affected in *Rara/g*-null mutants. These observations suggest that RAR α and RAR γ also exert a function in the mesodermal mesenchyme, as the supraoccipital bone and the lateral portion of the interparietal derive from the mesoderm (Chai and Maxson, 2006). Interestingly, the mesoderm-derived parietal bone is reduced in *Rara/g*^{NCC-/-} mutants, suggesting that it may be the result of a defect in the underlying NCC-derived meninges (Jiang et al., 2002b).

It is noteworthy that the otic capsule is normal in *Rara/g*^{NCC-/-} mutant mice, while severely malformed in *Rara/g*-null mutants (Lohnes et al., 1994). As this structure is derived both from mesoderm and NCC (Torres and Giraldez, 1998; O'Gorman, 2005) these observations suggest that the mesoderm can construct a normal otic capsule without involvement of RARs in the NCC.

The additional ablation of *Rarb* (yielding *Rara/b/g*^{NCC-/-} mutants) does not increase the severity or penetrance of the craniofacial skeletal defects, but induces severe ocular malformations (Matt et al., 2008 for a full description). This is accounted for by the functional redundancy between *Rarb* and *Rarg* in the periocular mesenchyme (Matt et al., 2005; Matt et al., 2008), rather than in the bone anlagen (present study). Thus, our data show that RAR α and RAR γ are the functional receptors transducing the RA signal in frontonasal NCC.

RAR α and RAR γ functions are fulfilled after the cessation of NCC migration

We show that NCC lacking all RARs are able to migrate into the frontonasal process and BAs, according to normal paths, as assessed from the distribution of *Crabp1* transcripts and from the NCC-specific β -galactosidase activity in *Rara/b/g*^{NCC-/-} mutants. Therefore, RARs are dispensable in NCC for their migration and survival until E10.5. However at E11.5, the nasal processes develop too widely apart and fail to fuse, eventually causing a wide midline cleft by E18.5. To understand at a cellular level the basis of the aberrant anatomy of E11.5 mutants, we analyse cellular processes underlying growth of the nasal prominences by measuring

proliferation and cell death. We could not detect any significant differences in proliferation. However, the widespread cell death normally occurring in E11.5 WT frontonasal mesenchyme is absent in *Rara/b/g*^{NCC-/-} and in *Rara/g*^{NCC-/-} mutants, indicating that RAR α and RAR γ mediate, without any contribution from RAR β , apoptosis during frontonasal mesenchyme maturation.

A failure of RA-induced physiological cell death in the frontonasal processes may be one of the mechanisms to account for the abnormal lateral position of the nasal processes. As the crucial merging of the facial primordial processes depends on their proper outgrowth, a change in the critical mass is likely to compromise this process. We cannot exclude the possibility that other mechanisms may also participate to this phenotype, however this impaired apoptosis constitutes an additional example illustrating a RAR-dependent cell-death process acting to sculpt the embryo. Analogous situations have been observed during heart, eye and limb development (Ghyselinck et al., 1998; Dupé et al., 1999a; Matt et al., 2005; Rodriguez-Guzman et al., 2007).

RA activating RAR α and RAR γ in frontonasal NCC is likely from (neuro)ectodermal origin

A local source of RA in the chick rostral head initiates a regulatory cascade that coordinates frontonasal process morphogenesis (Schneider et al., 2001). Similarly, in mice, expression pattern analyses have indicated that the RA-synthesizing enzyme ALDH1A2 is transiently expressed in the prospective forebrain neuroectoderm (Wagner et al., 2000; Smith et al., 2001), while ALDH1A3 is expressed later in the facial ectoderm (Mic et al., 2000) and ALDH1A1 is only expressed in the eye (McCaffery et al., 1991). In addition, craniofacial defects similar to those displayed by *Rara/g*^{NCC-/-} mutants have been described in *Aldh1a2/Aldh1a3* compound null mutants (Halilagic et al., 2006). These data, together with the present results, suggest that RA synthesized by ALDH1A2 then by ALDH1A3 in (neuro)ectodermal tissues may control the morphogenesis of the frontonasal process through diffusing towards the neighbouring RAR α - and RAR γ -expressing NCC-derived mesenchyme. In this context, RA would act in a paracrine manner to pattern the skeletogenic frontonasal NCC. Interestingly, a similar situation has been observed in the developing ocular region: RA is synthesized by the retinal pigmented epithelium, the retina and the corneal ectoderm [all derived from the (neuro)ectoderm], and it orchestrates eye morphogenesis through diffusing to the NCC-derived periocular mesenchyme where it activates RXR/RAR heterodimers.

Surprisingly, during these two processes RARs would have no autonomous function in the ectodermal tissues (Matt et al., 2005; Matt et al., 2008).

RARs are not required in NCC for the formation of branchial arches

The BA2 to BA4 are hypoplastic in *Rara/g*- and *Rara/b*-null mutants (Lohnes et al., 1994; Ghyselinck et al., 1997; Dupé et al., 1999b; Wendling et al., 2001), indicating that formation of these structures is RAR-dependent. The hypoplasia or absence of posterior BA in embryos deficient for RA signalling is correlated with changes in *Hoxa1*, *Hoxb1*, *Pax1* and *Pax9* gene expression in the endoderm (Dupé et al., 1999b; Wendling et al., 2000; Niederreither et al., 2003; Vermot et al., 2003). Since *Hoxa1* and *Hoxb1* are direct RA-target genes (Dupé et al., 1997; Huang et al., 1998), and considering that BA endoderm is a primary target tissue of RA-induced teratogenesis (Matt et al., 2003; Vieux-Rochas et al., 2007), a model in which RARs in the endoderm regulate the formation of the pharyngeal pouches has been proposed (Mark et al., 2004; Vieux-Rochas et al., 2007).

As *Pax1* is severely down-regulated in the endoderm of *Rara/g*- and *Rara/b*-null mutants, we have tested its expression in *Rara/b/g^{NCC-/-}* and observed a normal expression pattern. In addition, by observing histological section of E9.5 embryos, we show that the formation of BA2 to BA4 is essentially not affected in *Rara/b/g^{NCC-/-}* embryos. Therefore, these two data unambiguously demonstrate that NCC do not represent the main target tissue of RARs during BA formation. Along the same lines, we have previously shown in *Rara/b*-null mutants that RAR α and RAR β are together essential for the organization of the NCC-derived post-otic cranial nerve and of their distal ganglia (Dupé et al., 1999b; Wendling et al., 2001). Surprisingly, cranial nerves of *Rara/b/g^{NCC-/-}* mutants are not affected, thereby indicating that RAR do not fulfil cell-autonomous functions in the patterning of neurogenic NCC.

RARs are largely dispensable in NCC for the early morphogenesis of BA-derived structures

In *Rara/g*-null mutants, the formation of BA1 is unaffected (Wendling et al., 2001). Accordingly, BA1-derivatives are all identifiable although some of them are misshapen. We previously proposed that this can be accounted for by the severe malformations of the neighbouring (i) mid-facial skeletal elements (as to defects of the maxillary, squamosal and alisphenoid bone) and (ii) otic capsule (as to defects of the malleus, meckel, incus cartilages). In addition, more anterior structures derived from the mandibular portion of BA1 (i.e.,

dentary bone, mandibular molars and incisors) are unaffected (Lohnes et al., 1994; Ghyselinck et al., 1997). In *Rara/b*-null mutants, the cranium is essentially normal (Lohnes et al., 1994; Ghyselinck et al., 1997).

The present study shows that in *Rara/b/g*^{NCC/-} mutants, maxillary BA1-derived structures including the maxillary, alisphenoid and squamosal bones are well recognizable, but generally reduced, similar to *Rara/g*-null mutants. The presence of these misshapen bones may illustrate a RAR requirement in NCCs during later developmental process such as the modelling of skeletal elements. Indeed, the enlargement of the short process of the incus displayed also by *Rara*^{NCC/-} mutants demonstrates that RARs are important in NCC for sculpting the morphology of skeletal element.

In contrast, in *Rara/b/g*^{NCC/-} mutants, mandibular BA1-derived structures including Meckel's cartilage and malleus appear normal. Thus, this observation suggests that the severely malformed malleus and Meckel cartilages previously described in *Rara/g*-null mutants may be explained by the severe malformation of the otic capsule. In contrast, the gonial and tympanic bones are still distorted in *Rara/g*^{NCC/-} mutants, it suggests that RAR α and RAR γ have a function in modelling these two bones. Since we observe discrete cranial skeletal irregularities, we propose that early morphogenesis of BA1-derived structures does not rely on RARs in NCC. Nevertheless, RARs in NCC are important later for sculpting the morphology of the skeletal components of the head. It may represent an important mechanism for driving morphological changes in facial architecture during evolution. Such a mechanism is reinforced by experiments implicating elevation of RA that also lead to skull and face bone defects (Vieux-Rochas et al., 2007; Maclean et al., 2009). In both *Rara/g*- and *Rara/b*-null mutants, the alisphenoid and incus, which are derived from the maxillary process of BA1, are fused through a supernumerary piece of cartilage to form a pterygoquadrate element that has been lost during mammalian evolution (Mark et al., 1998). Intriguingly, the pterygoquadrate is never observed in *Rara/b/g*^{NCC/-} mutants, suggesting that RARs are required in a tissue (or a cell-type) distinct from NCC to allow involution of this atavistic structure of the upper jaw (Mark et al., 1998).

In contrast to BA1, the initial formation of BA2 is impaired in *Rara/g*-null mutants (Wendling et al., 2001), and BA2-derived elements (i.e., the stapes, hyoid lesser horn, styloid bone and pinna) are consequently severely malformed (Lohnes et al., 1994). We show in the present study that BA2 is properly formed in *Rara/b/g*^{NCC/-} embryos and that none of its derivatives

are affected. Thus, RAR-depleted NCC can differentiate normally into cartilage and their initial patterning is unaltered. Altogether these findings demonstrate that RARs are dispensable in NCC for the formation of BA2-derived structures, suggesting thereby that they predominantly exert their role in a cell population distinct from NCC.

Rara/b-null and *Rara/g*-null mutants display the complete set of defects that can be generated in the chick by surgical ablation of post-otic NCC normally migrating in BA3-BA4, namely aberrant patterning of great arteries, together with persistent truncus arteriosus (PTA) and interventricular septal defects, and thymus and parathyroid gland agenesis or ectopia (Hutson and Kirby, 2003). Post-otic mesenchymal NCC also contribute to the body of the hyoid bone and the thyroid cartilages (Kontges and Lumsden, 1996; Santagati and Rijli, 2003). Interestingly, the defects in the patterning of great arteries, which are severe and consistently observed in *Rara/b*- and *Rara/g*-null mutant (Mendelsohn et al., 1994; Ghyselinck et al., 1997), occur with a low penetrance in *Rara/b/g*^{NCC-/-} fetuses. Furthermore, BA4 appears to be the most affected BA, since the defects in great arteries observed (i.e. the right aortic arch and the retroesophageal subclavian artery), result from remodelling of the 4th aortic arch. In addition, all the neck glands (i.e., thymus, thyroid and parathyroids) are present in *Rara/b/g*^{NCC-/-} fetuses (Ghyselinck et al., 1997). Finally, contrary to the situation in *Rara/b*-null mutants, the shapes of the hyoid bone and of the laryngeal cartilages are normal in *Rara/b/g*^{NCC-/-} fetuses, with the notable exception of a fusion between the greater horn of the hyoid bone and the rostral horn of the thyroid cartilage, which are also BA4-derived structures.

The present work thus indicates that RARs have a cell-autonomous function in NCC during the development of a few given post-otic BA-derived structures, mainly those derived from the BA4. However, the overall study clearly shows that RAR-dependent formation of most of the post-otic BA-derived structures does not rely on the presence of RARs in NCC. A previous study, based on pharmacological study (Matt et al., 2003), favoured a model in which the main function of RARs in BA2 to BA6 was taking place in the pharyngeal endoderm; the present genetic study clearly demonstrates its physiological relevance.

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FIGURE LEGENDS

Fig. 1: Comparison of neural crest cells distribution in E9.5 wild type (WT) and *Rara/b/g*^{NCC-/-} mutant embryos. (a-f) Distribution of β -galactosidase activity in mice harbouring both *R26R* and *Wnt1-Cre* transgenes. The β -galactosidase staining in WT and *Rara/b/g*^{NCC-/-} is undistinguishable in (a,b) whole embryo preparations and (c-f) histological sections. (g-n) In situ hybridization analyses: (g,h) *Crabp1* and (i,j) *Dlx5* expressions are normal in branchial arches of *Rara/b/g*^{NCC-/-} mutants, while (k,l) expression of *Pax1* demonstrates that the branchial arches are normally segmented. (m-p) Analysis of cranial nerves using: (m,n) *Sox10* as a molecular marker and (o,p) an anti-neurofilament antibody demonstrate a normal pattern of the cranial nerves and associated ganglia in *Rara/b/g*^{NCC-/-} mutants. Abbreviations. A1-A3: aortic arches 1-3; B1-B6: branchial arches 1-6; EN: BA endoderm; EC: BA ectoderm; F: frontonasal mesenchyme; FB: forebrain; H: heart; M: mesoderm; NP: nasal process; P1-P3: pharyngeal pouches 1-3; G5: trigeminal ganglion; G7/8: facial-acoustic ganglion; N9: 9th cranial nerve; N10: 10th cranial nerve.

Fig. 2: Craniofacial defects in *Rara/b/g*^{NCC-/-} mutants. (a-d) Heads of (a) WT, (b) *Rara/g*^{NCC-/-} and (c,d) *Rara/b/g*^{NCC-/-} E18.5 fetuses. White arrowheads (in a-b) point to the eye and the asterisk indicates the absence of externally visible eye structures (Matt et al., 2008). Black arrowheads (in b-d) point to the median facial cleft. (e-l) Skeletal preparations of heads from E18.5 WT, *Rara/b/g*^{NCC-/-}, *Rarg*^{NCC-/-} and *Rara*^{NCC-/-} mutants (genotypes indicated in panels). (e,f) Lateral view; (g,h) Ventral view. In (f) the asterisk indicates the hypoplastic frontonasal skeleton. In (i), comparative view of dissected bones. Interparietal and dentary bones are normal. Mutants have smaller parietal, frontal and squamosal bones. Tympanic and gonial bones are distorted. In (k) the asterisk indicates the severely reduced presphenoid bone of the *Rarg*^{NCC-/-} mutant. In (l) note the lengthening of the caudal process (CI) of the *Rara*^{NCC-/-} mutant incus. Abbreviations: AL: alisphenoid bone; BO: basioccipital bone; BS: basisphenoid bone; CI: caudal process of the incus; D: dentary (mandibular) bone; E: exoccipital bone; EE: external ear; F: frontal bone; G: gonial; I: interparietal bone; N: nasal bone; O: otic capsule; P: parietal bone; PS: presphenoid bone; PX: premaxillary bone; S: supraoccipital bone; SI: stapedial process of the incus; SQ: squamosal bone; TB: tympanic bone; X: maxillary bone.

Fig. 3: Cardiovascular and glandular defects in *Rara/b/g*^{NCC-/-} mutants. Histological sections of the heart outflow tract region of E14.5 fetuses show separated aortic (AO) and pulmonary (PT) trunks in (a) WT, whereas (b) *Rara/b/g*^{NCC-/-} mutants exhibit an unseptated outflow tract (i.e., a persistent truncus arteriosus; PTA). (c-e) Histological sections through the thyroid gland region of (c) WT and (d-e) *Rara/b/g*^{NCC-/-} E18.5 fetuses, showing (d) a lack of parathyroid glands within the thyroid glands in the mutant and (e) their ventral ectopia. (k) The black asterisk indicates an ectopic cartilage connecting the body of the hyoid bone to the thyroid cartilage. Abbreviations: AO: aorta; H: body of the hyoid bone; LH: lesser horn of the hyoid bone; PT: pulmonary trunk; PTA: persistent truncus arteriosus; PTh: parathyroid glands; T: thyroid cartilage; Th: thyroid gland.

Fig. 4: E11.5 *Rara/g*^{NCC-/-} and *Rara/b/g*^{NCC-/-} mutants present a median facial cleft. (a,b) Heads of E11.5 (a) WT and (b) *Rara/g*^{NCC-/-} embryos. Double-headed black arrows point to the distance between the nasal placodes. FB: forebrain; NP: nasal process.

Fig. 5: Ablation of RARs in neural crest cells decreases apoptosis in the frontonasal mass. (a,e) Distribution of proliferating cells (as assessed by phosphohistone H3 immunodetection) in E11.5 (a) WT and (e) *Rara/b/g*^{NCC-/-} embryos. (b-d, f-h) Distribution of apoptotic cells (as assessed by TUNEL assays) at E10.5 (b,f) and E11.5 (c,d,g,h). Brackets (in c and g) indicate clusters of apoptotic cells in the midline region. Black arrowheads (in b,d) indicate clusters of apoptotic cells located in the dorsal frontonasal area of WT embryos. Abbreviations. F: frontonasal mesenchyme; FB: forebrain; GE: ganglionic eminence; H: heart; M: maxillary primordium; MD: mandibular primordium; N: nasal cavity; NP: nasal process.

Table 1: Malformations of cranial skeletal elements in *Rar*^{NCC-/-} mutants compared to the *Rar*-null compound mutants observed at E18.5. Legend. *: abnormality present in a majority of the mutants; #: abnormality fully penetrant; (1) Lohnes et al., 1994 and Ghyselinck et al., 1997.

	Genotypes of <i>Rar</i>^{NCC-/-} and <i>Rar</i>-null compound mutants showing similar defects	
Abnormalities	Compound <i>Rar</i>^{NCC-/-} mutants	Compound <i>Rar</i>-null mutants⁽¹⁾
Posterior structures of the head <ul style="list-style-type: none"> • Supraoccipital and interparietal bone reduced • Parietal bone reduced • Exencephaly 	0 <i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#} 0	<i>Rara/g</i> [#] <i>Rara/g</i> [#] <i>Rara/g</i>
Midfacial skeletal elements <ul style="list-style-type: none"> • Frontal bone, premaxillary bone and nasal capsule severely reduced. • Agenesis of the presphenoid bone and metoptic pillar. • Pila antotica 	<i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#} <i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#} , <i>Rarg</i> ^{NCC-/-#} <i>Rara/b/g</i> ^{NCC-/-*} , <i>Rara/g</i> ^{NCC-/-*}	<i>Rara/g</i> [#] <i>Rara/g</i> [#] , <i>Rarg</i> [*] <i>Rara/g</i> [#]
First branchial arch-derived elements <u>Maxillary derivatives :</u> <ul style="list-style-type: none"> • Maxillary, squamosal and alisphenoid reduced • Enlarged short process of the incus • Pterygoquadrate cartilage <u>Mandibular derivatives :</u> <ul style="list-style-type: none"> • Severely malformed Malleus and Meckel cartilage • distorted gonial bone 	<i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#} <i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#} , <i>Rara</i> ^{NCC-/-#} , <i>Rara/b</i> ^{NCC-/-#} , 0 0 <i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#} , <i>Rara/b</i> ^{NCC-/-} ,	<i>Rara/g</i> [#] <i>Rara</i> , <i>Rara/g</i> [#] , <i>Rara/b</i> <i>Rara</i> , <i>Rara/g</i> [#] , <i>Rara/b</i> <i>Rara/g</i> [#] <i>Rara/b/g</i> [#] , <i>Rara/g</i> [#] , <i>Rara/b</i> ,
• Mild hypoplasia of the tympanic bone	<i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#}	<i>Rara/g</i>
Second branchial arch-derived elements <ul style="list-style-type: none"> • Stapes, styloid and lesser horn of the hyoid bones highly malformed • Agenesis of the pinna (external ear auricle) 	0 0	<i>Rara/g</i> [#] <i>Rara/g</i> [#]
Abnormal laryngeal cartilages <ul style="list-style-type: none"> • Body of the hyoid bone fused to the thyroid cartilage • Malformed arytenoid and cricoid cartilage 	<i>Rara/b/g</i> ^{NCC-/-#} , <i>Rara/g</i> ^{NCC-/-#} 0	<i>Rara/g</i> [#] <i>Rara/g</i> [#] , <i>Rara/b</i> [#]
• small otic capsule	0	<i>Rara/g</i> [#]

Table 2: Cardiovascular and glandular abnormalities in *Rar*^{NCC-/-} mutants. Legend. + : presence of ectopic parathyroid gland, percentage not determined. (1) Lohnes et al., 1994; (2) Ghyselinck et al., 1997. Cardiovascular defects were evaluated at E14.5 and E18.5 whereas glandular defects were evaluated at E18.5.

Abnormalities	Mutant genotypes				
	<i>Rara/g</i> ^{NCC-/-}	<i>Rara/b/g</i> ^{NCC-/-}	<i>Rara/b</i> ^{NCC-/-}	<i>Rara/g</i> ⁽¹⁾	<i>Rara/b</i> ⁽²⁾
• Persistent truncus arteriosus	0/6	3/7	0/6	100%	100%
• Abnormal aortic arch 4	2/6	3/7	0/6	100%	100%
• High ventricular septal defect	0/6	3/7	0/6	100%	100%
• Thymus agenesis or ectopia	0/3	0/3	0/2	100%	50%
• Thyroid hypoplasia or ectopia	0/3	0/3	0/2	80%	20%
• Parathyroid gland ectopia	0/3	3/3	0/2	+	+